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Clinically relevant sequence-based genotyping of HBV, HCV, CMV, and HIV

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Abstract

The term 'genotyping' describes the genetic characterization of a genome. The genotype analysis is performed to identify mutations that differentiate one individual or strain from another. The mutations may confer resistance to specific antiviral drugs or they may simply allow classification of a strain as to 'type' and 'subtype'. There are four human viruses for which genotype information is clinically useful. Hepatitis B virus (HBV) infections are being treated with antiretroviral drugs and resistance after prolonged treatment is common. Since HBV cannot be cultured, the only method of detecting resistance-conferring mutations in the genome is a genotypic analysis. Hepatitis C virus (HCV) infection can be cured by treatment with the combination of interferon and ribavirin but certain strains of virus are more resistant to treatment than others. The current recommendations are that all HCV type 1 infections be treated for 12 months whereas other types may be successfully treated in 6 months. Since interferon treatment may have significant side effects, the determination of HCV genotype is an important aspect of this therapeutic regimen. Treatment of cytomegalovirus (CMV) disease with nucleoside analogues occasionally results in resistant virus with mutations in the phosphotransferase gene (UL97) and/or the DNA polymerase gene (UL54) that can be tested with phenotypic or genotypic assays. Since CMV grows very slowly, it may be more clinically useful to perform a rapid genotypic assay although only the UL97 gene can be efficiently genotyped. Finally, the virus for which genotyping has become the standard of care, human immunodeficiency virus type 1 (HIV-1) can now be genotyped routinely by many clinical virology labs experienced with molecular amplification methods and automated DNA sequencing technology. All currently-available antiretroviral drugs are directed against either the protease or reverse transcriptase genes of HIV-1 and the mutations within these genes that confer resistance have been well described. Sequence-based genotyping methods are not necessarily the best approach for routine genotyping of these four viruses, but sequencing is the gold standard from which other methods are developed and against which they are compared.

Abbreviations: HBV-hepatitis B virus; HCV-hepatitis C virus; CMV-cytomegalovirus; HIV-1-human immunodeficiency virus type 1; PCR-polymerase chain reaction; RT-reverse transcriptase; FCV-famciclovir; GCV-ganciclovir; SVR-sustained virologic response; PRA-plaque reduction assay.

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1. Introduction

As virologists have become progressively more efficient at detecting and identifying viruses and as antiviral therapies have become more effective and more widely used, it has become interesting and important for the clinical virology lab to be able to perform genotypic analyses pursuant to typing and subtyping of viruses for epidemiologic purposes and for antiviral drug susceptibility testing. 'Genotyping' in the context of clinical virology refers to the characterization of all or part of a viral genome with regards to the presence of 'mutations' or changes in the genetic code of that particular organism compared to a 'reference' or 'wild type' strain of the same organism. Genotypic analysis of viral genomes has become an important aspect of clinical virology. The foundation upon which all genotypic analyses are built is the nucleotide sequence of the viral genome. Once this sequence is known, other methods which utilize the information can evolve into techniques for more efficient detection of a specific mutation or set of mutations. In some cases however, sequencing remains as the most efficient and widely-used method for routine genotyping.

The era of the polymerase chain reaction (PCR) and automated sequencing has opened the door to numerous possibilities in the clinical virology lab. Today virologists have techniques available for molecular analysis that were beyond our wildest dreams a mere 15 years ago. For example, we can quickly and routinely determine the nucleotide sequence of the entire protease and reverse transcriptase (RT) genes of a clinical specimen containing HIV and thus detect mutations that confer antiretroviral drug resistance and aid in directing patient therapy. This and other uses of sequencing methodology for determination of viral genotype are important today and will become more important in the molecular-oriented clinical virology lab of the future.

1.1. Genotype vs. phenotype

With regards to drug susceptibility testing, whereas a genotype assay detects mutations in the genome that may confer drug resistance, phenotyping measures the actual ability of the virus to grow in the presence of various chemical compounds or known antiviral drugs. Thus, both assays have the same purpose but they arrive at their respective results from different directions. For discovery of new antiviral drugs, obviously one must employ phenotypic assays to test their ability to inhibit growth of viruses in culture and only after considerable research and clinical experience might we discover the exact location of mutations in the viral genome that confer resistance to a particular drug. Phenotypic assays have the advantage of measuring the growth characteristics of a virus even in the context of a complex assortment of mutations in which it may be very difficult to sort out and identify their individual actions and collective interactions. Phenotypic assays have the obvious disadvantage that they cannot be used with viruses whose growth cannot be measured in culture, as in the case of HCV. Genotyping, on the other hand, can be a fairly simple, straightforward procedure that yields clinically useful information at a reasonable cost and in a short amount of time and is routinely performed on the nucleic acid extracted from patient plasma, thus precluding the necessity of an in vitro culture. The major requirement for interpretation of genotypes for resistance testing is that the location of mutations that confer resistance must be known and there should be some concept of which mutations confer what level of resistance.

1.2. Sequencing vs. other methods

Sequencing is not the only method for determination of a genotype, whether it be for viral typing and subtyping (Arens, 1999) or for resistance testing (Erice, 1999a). However, all geno-

typic methods for evaluation of drug resistance rely on sequence information to identify the nucleotides of interest. Other methods used to query specific positions in a viral genome include restriction enzyme digestion of PCR products (Schlesinger et al., 1995; Takayama et al., 1996), primer-specific PCR (Zou, 1997), reverse hybridization of PCR products to probes on nitrocellulose strips (Le Pogam et al., 1998) (the line probe assay, LiPA™; Innogenetics, Inc.), hybridization to microarrays or silicon chips containing thousands of specific probes (Chambers et al., 1999) and cleavage of probes by endonucleases with specific requirements for the tertiary structure of hybrids (Cleavase™; (Oldenburg and Siebert, 2000)). These methods may be much easier to perform than sequencing since they were developed for a specific purpose and they only detect mutations at certain locations within a specific genome. Many of them may assume a format that is easily adapted to the routine of a clinical virology lab. Thus, these techniques yield information on the presence or absence (and possibly the relative amounts of mutant vs. wild type) of mutations at one or more specific locations but they give no information about other nucleotide positions in the genome

1.3. The basics of sequencing methodology

Various methods of nucleotide sequencing have been described over the past 30 years but the Sanger method (Sanger et al., 1977) has emerged as the most widely used technique (Fig. 1). This method makes use of the fact that DNA polymerases can only initiate DNA synthesis at the 3′ end of a primer hybridized to a target DNA (Fig. 1B) and the fact that dideoxynucleotides, when added to a DNA polymerase reaction mixture in low concentrations, will randomly terminate the growing oligonucleotide chains (Fig. 1C). Thus, all DNA made in a reaction with only one specific primer and one target will be initiated at the 3′ end of that primer. And, all growing oligos, when randomly terminated by a dideoxynucleotide at a specific length, will be terminated with the same base. For example, oligos terminated at the 10th base from the primer (Fig. 1C) will be terminated with whatever base is complementary to the 10th base (from the primer) in the target DNA. The oligo generated by this method will actually be covalently attached to the primer so its length will be the sum of the primer bases plus the bases added by the in vitro DNA polymerase reaction. If the primer is labeled for detection, or if the four dideoxyterminators are all labeled with the same identifier (e.g. ^{32}P), then four different reactions are run and the oligos from each reaction are separated in four different lanes of a polyacrylamide gel and visualized by autoradiography as in the classic Sanger procedure. Sequencing electrophoresis gels can separate oligos that differ by only one base in length.

Recent advances in sequencing technology have simplified this process considerably. We now use primers or terminators labeled with four different fluorescent dyes, one dye is always associated with a particular dideoxyterminator so that oligos terminated with that dye can be identified by their characteristic fluorescence color in the polyacrylamide gel. As oligos are subjected to electrophoresis in the gel, they pass a laser scanner and the peaks of fluorescence (one color for each of the four dye-terminators) are detected and recorded by a dedicated computer as peaks and valleys in the scan. The computer records the color of the peaks, translates the color of each peak into a base (A, C, G or T) and records the linear sequence of the bases as the 'genotype.' Modifications such as 'cycle-sequencing' in a thermal cycler, new thermostable sequencing enzymes, new dyes and commercial computer software continue to refine the technology to improve ease of use and efficiency (Fig. 2). Read lengths of 500–700 bases from one primer are fairly routine with today's dyes and DNA polymerase enzymes. With sequencing primers spaced every 400–600 bases, genomes of any length can be sequenced. The basic requirements for a sequence that is accurate and easy to read are shown in Table 1.

1.4. Clinically useful viral genotypes-which, why and how?

Virtually all viruses that we know about today have been sequenced. In fact, some new viruses were discovered by sequencing of their genetic

material even before they were described as new entities (e.g. hepatitis C virus (HCV) and the Kaposi's sarcoma-associated virus, HHV-8). For all practical purposes, there are only four viruses for which a genotypic analysis is clinically useful, HBV, HCV, CMV and HIV. This is mostly a function of the fact that there are not many viral infections that can be treated with antiviral drugs and thus there are few opportunities for viruses to become resistant to antiviral drugs. Herpes simplex virus (HSV) and influenza virus can be treated with antiviral drugs but at the present

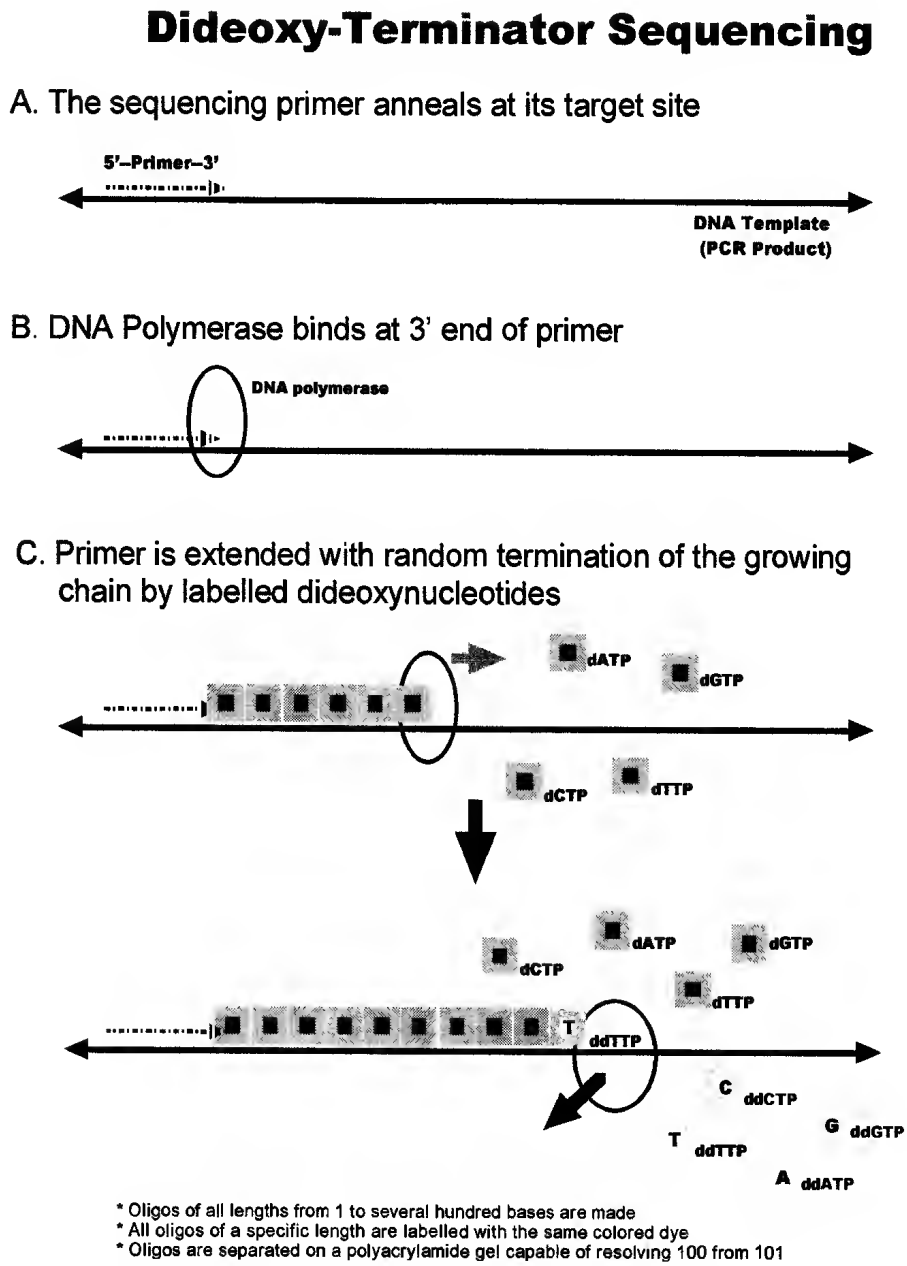

Fig. 1. The steps of the Sanger method of dideoxy — terminator sequencing. The basic principles of the method are: (1) the sequencing primer can only bind at one location on the target DNA; (2) DNA polymerase can only bind and initiate synthesis at the 3′ end of the bound primer; (3) dideoxynucleotides present in the reaction at low concentrations are randomly incorporated into a growing chain and thereby cause termination of that oligonucleotide; and (4) oligonucleotides of varying lengths are separated in high-resolution polyacrylamide gels.

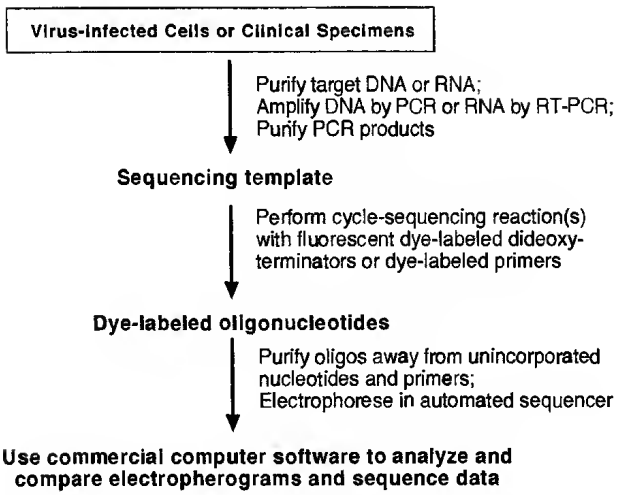

Fig. 2. Overview of the process of automated cycle sequencing. (From (Arens, 1999) reprinted with permission).

time gentoypic analysis of these viruses is either not useful (Lee et al., 2000) or not practical.

1.5. HBV

As many as 5% of the world's population may be chronically infected with HBV and this infection is among the leading causes of death worldwide, contributing to an estimated 40 million deaths each year (Lee, 1997). Interferon-alpha is licensed for treatment of HBV but fewer than 40% of patients respond to this immunotherapy. Since HBV and HIV have closely related reverse transcriptase enzymes, drugs that are effective against HIV have recently been investigated and found to have significant clinical activity against HBV.

Table 1
Requirements for a 'good' sequence

Purified nucleic acid target for PCR or RT-PCR
Purified amplified product
Free of all PCR primers (if multiple primers are present, the sequence cannot be interpreted)
A single band on agarose gel is desirable but not necessary if no alternative primer binding sites are present
Highly purified sequencing primers
Stringent electrophoresis conditions (gel, buffer, temperature, gel length, dyes, etc)
Appropriate computer software to properly interpret the electropherogram data

1.5.1. Virus replication

The replication of HBV is a complex process involving the basic steps of: (1) entry into the host cell; (2) uncoating of the virion; (3) 'repair' in the nucleus of the virion DNA to form covalently closed circles (or ccc DNA) by the DNA-dependent DNA polymerase; (4) transcription to produce plus-strand RNA; (5) encapsidation of the plus-strand RNA; (6) reverse transcription within the virion of RNA by the RNA-dependent DNA polymerase activity to produce minus-strand DNA; (7) partial (incomplete) synthesis of plus-strand DNA by the DNA-dependent DNA polymerase activity within the virion; and (8) assembly into complete virions and secretion out of the cell (Ganem, 1996). Thus, the HBV polymerase has several activities to accommodate this complex replicative mechanism of the virus, including RNA-dependent DNA polymerase, DNA-dependent DNA polymerase and RNase H activity to remove the RNA moiety of an RNA:DNA hybrid. These various activities are distinct targets for antiviral drugs, several of which may be used together to inhibit replication at different stages.

1.5.2. Antiviral drugs

Nucleoside analogues such as lamivudine (3TC), famciclovir (FCV) and ganciclovir (GCV) are potent inhibitors of the HBV polymerase both in vitro and in vivo (Zoulim and Trepo, 1998). Lamivudine was shown nearly 10 years ago to inhibit HBV replication in vitro (Doong et al., 1991) and subsequent clinical trials have proven its usefulness in vivo (Lai et al., 1998; Dienstag et al., 1999). Treatment of chronic hepatitis B in the USA for 1 year showed that patients treated with 3TC were more likely than placebo to have a histologic response (52 vs. 23%), sustained suppression of HBV DNA in serum to undetectable levels (44 vs. 16%) and sustained normalization of serum ALT levels (41 vs. 7%) (Dienstag et al., 1999). Lamivudine is currently the only nucleoside analogue approved for treatment of HBV infection. However, several others are in the testing pipeline. Famciclovir (pro-drug of penciclovir) inhibited production of HBV in a producer cell line (Korba and Boyd, 1996) and was highly selective in its inhibition of HBV polymerase over cellular

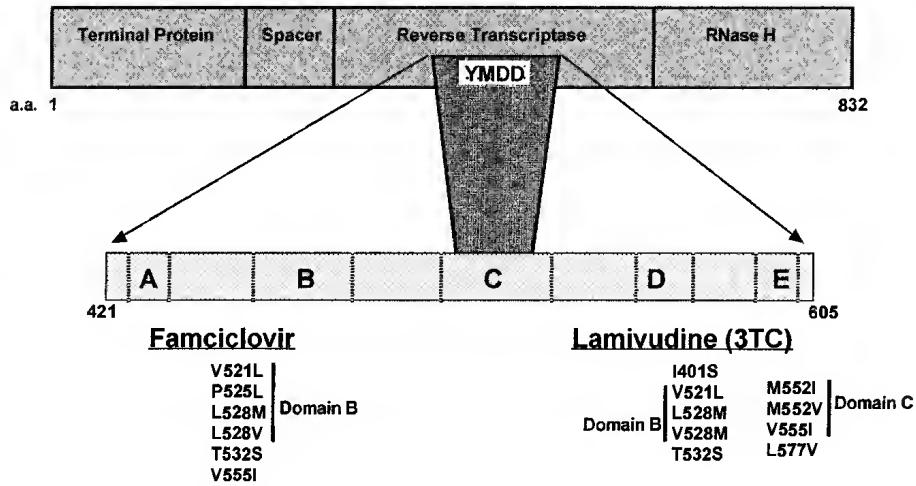

Fig. 3. The HBV polymerase and mutations associated with resistance to nucleoside analogues. Within the reverse transcriptase region of the polymerase gene are the five domains A to E. Mutations at certain codons (see lower part of Figure) in the B and/or C domains have been shown to confer resistance to famciclovir or lamivudine.

DNA polymerases (Shaw et al., 1996). An early pilot study to assess effectiveness (Main et al., 1996) and a more recent 16-week dose-finding clinical trial (Trepo et al., 2000) have borne out the clinical usefulness of FCV in HBV-infected individuals. Longer studies with this drug are clearly warranted. Finally, the drug of choice for treatment of CMV infections, ganciclovir, is also effective against HBV. In a long-term trial that began in the early 90s, intravenous GCV was used for a median of 10 months to treat liver transplant patients with HBV infection (Roche et al., 1999). No resistance to GCV was documented. The results of this trial were encouraging but the first-line drugs discussed above are easier to administer and less toxic and thus GCV is a possible contender for treatment of recurrent HBV in the face of high-level resistance to other drugs.

1.5.3. Relevant mutations

Early investigations into the breakthrough viremia of a patient on 3TC therapy revealed mutations at codons within the active site of the polymerase (Tipples et al., 1996). The YMDD motif (Fig. 3) is present in both the HIV and HBV polymerases and a single mutation within these amino acids (codon 184 in HIV and codon 552 in HBV) can confer high level resistance to 3TC in either virus. Lamivudine resistance was documented in 4 of 14 immunocompetent patients with chronic HBV infection treated for 26 weeks (Honkoop et al., 1997). In another trial of HIV-infected patients also infected with HBV, all 19 patients became HBV DNA negative after a median of 16 months treatment but seven developed breakthrough DNAemia after 8–34 months following cessation of treatment (Fontaine et al., 1999). Breakthrough was more likely after longer treatment times, thus boding poorly for patients requiring extended periods of treatment to clear viral DNA from serum. This was documented further in a study of HIV–HBV-infected patients given 3TC for 2 years (Benhamou et al., 1999) in which it was estimated that the emergence of 3TC-resistant HBV occurred in 20% of patients per year. Resistance to famciclovir has been documented and the associated codons within the polymerase gene have been identified outside of the YMDD motif (Fig. 3). Early reports (Aye et al., 1997; Wolters et al., 1998) described 3TC treatment failures that did not respond to FCV and had mutations within the YMDD motif (M = codon 552) as well as a second mutation at codon 528 in the B-domain of the HBV polymerase. Others found FCV failure to be independent of mutations in the YMDD motif and identified several other regions of the gene responsible for FCV resistance (Gunther et al., 1999)

(see also Fig. 3). Recent in vitro enzymatic assays have investigated the relative effects on the inhibition constants of the five most commonly seen mutations in FCV-treated patients, V521L, P525L, L528M, T532S and V555I (Xiong et al., 2000). These studies showed that V555I confers the highest resistance to FCV, with V521L and L528M mutations resulting in somewhat lower levels of resistance. Additionally, the 525 and 555 mutations resulted in lower sensitivity to 3TC.

1.5.4. Sequencing methods

Primers for use in amplification and sequencing of the HBV pol gene are published. Allen et al (Allen et al., 1998) identified three sets of PCR primers and the appropriate corresponding sequencing primers for amplification and sequencing of the pol gene. They amplified products of 487, 567 or 1076 base pairs (bp) spanning the YMDD motif and then used 3 or 4 sequencing primers to cover the amplicons in both directions. All mutated amino acids that they observed in 3TC-treated patients were between codons 430 and 577, inclusive, so that in the future only these 147 codons (441 base pairs) may need to be sequenced in order to sample all apparent mutation sites. More recent indications are that the truly significant mutations for both 3TC and FCV resistance are between codons 528 and 555, inclusive, which narrows even further the region of interest for genotypic analysis. In another published method, a 667 bp PCR product was amplified and sequenced with 2 inner primers covering a region of 380 bp or 126 codons surrounding the YMDD motif (Thibault et al., 1999). The nine HIV–HBV coinfected patients who failed 3TC treatment in this study had HBV pol mutations only at codons M552V and M528L ($n = 7$), codons M552I and M528L ($n = 1$) and M552I only ($n = 1$). Thus, it appeared as though the mutations at these two locations were entirely responsible for the 3TC resistance and HBV breakthrough. Detection by sequence-based genotyping of these mutations separated by only 78 bp is an easy task by today's standards.

At the present time, treatment of HBV with antivirals does not seem to be a huge enterprise. However, it is estimated that as many as 350 million people worldwide may be infected with HBV and this presents the daunting possibility that a huge number of people may be candidates for therapy if effective, durable treatments are identified. Genotypic monitoring for resistance to antiviral drugs will be a natural consequence of such treatment.

1.6. HCV

HCV infection is estimated to affect over 300 million people worldwide and nearly 4 million Americans, about 15–20% of whom, if untreated, will develop chronic liver failure and cirrhosis. HCV infection engenders a strong immune response but is frequently able to escape and cause chronic infection. The ability to escape antibody and cytotoxic T-lymphocyte (CTL) responses is likely a function of the propensity of the virus during the course of infection to generate a highly variable population or quasispecies within the host. Virus is produced at a very high rate, on the order of 10^{12} virions per day, and virions have a very short half-life of about 2.7 h (Neumann et al., 1998). HCV is not directly cytopathic and liver damage is the result of immune-mediated mechanisms. Host cofactors play an important role in the ultimate outcome of the infection and disease (Boyer and Marcellin, 2000).

1.6.1. Rationale for genotyping

In recent clinical trials, the treatment combination of interferon alfa-2b and ribavirin resulted in a sustained virologic response (SVR, i.e. HCV was not detectable at 24 weeks after cessation of treatment) in 31–33% of patients treated for 24 weeks and in 38–43% of patients treated for 48 weeks (McHutchison et al., 1998; Poynard et al., 1998, 2000). The response to combination therapy of patients with HCV types 2 or 3 who were treated for 6 or 12 months were similar (62–71% response) regardless of their initial viral load (Poynard et al., 2000). On the other hand, type 1 HCVs are somewhat refractory to treatment; combination therapy for 6 or 12 months resulted in 26 or 33% SVR, respectively, in patients with initially low ($< 3.5 \times 10^6$ c/ml) viral loads or 10 or 27% SVR, respectively, in patients with initially

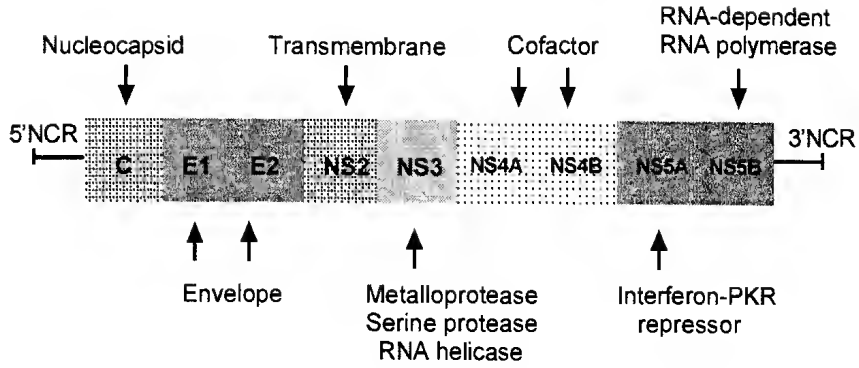

Fig. 4. The genome of HCV. A single RNA transcript is translated into a polyprotein which is then cleaved into 10 separate functional proteins.

high ($>3.5 \times 10^6$ c/ml) viral loads (Poynard et al., 2000). Combination therapy is also effective in about 45% of patients who have relapsed after initial interferon monotherapy.

A re-analysis of two large multicenter randomized HCV treatment trials was recently undertaken to identify factors associated with a favorable viral response (Poynard et al., 2000). The factors associated with a favorable response were: younger vs. older age, female vs. male gender, no or limited liver fibrosis vs. extensive fibrosis, low vs. high viral load and HCV genotypes 2 or 3 vs. genotype 1. Thus, genotype analysis should be part of treatment algorithms at some point in the process. The recommendation of a recent International Consensus Conference (Poynard et al., 2000) is that there is no need to determine viral load or viral genotype on patients who are HCV qualitative PCR positive after 24 weeks of combination therapy since they are most likely to be non-responders even if they have 4 or 5 of the favorable response factors. However, for patients who are PCR negative at 24 weeks and therefore likely to enjoy an SVR, physicians need to know the initial viral load and the genotype so as to decide whether to continue treatment for an additional 24 weeks. Thus, viral load and genotype should be performed retrospectively on saved samples in order to aid with this decision. Very high-risk patients, men over 40 years and with fibrosis should be continued on therapy regardless of the viral load and genotype results (Poynard et al., 2000). Evaluation of the early (first few months) response to combination therapy has been shown to be a strong independent predictor of SVR (Castro et al., 2000), but this concept is controversial.

1.6.2. The HCV genome

The organization of the HCV genome is shown in Fig. 4. The genome is translated into a large polyprotein which is subsequently cleaved into smaller, functional subunits. The 5′-untranslated region (UTR; also called non-coding region or NCR), the nucleocapsid or core (C) gene, the envelope (E1) region and the replicase (NS5B) region have most often been used for analysis of genotype and subtype (Table 2). HCV types are assigned by phylogenetic tree analysis of many sequences from a certain genomic region. The tree analysis has the ability to group sequences together based on specific differences within the context of their general similarity. Thus, as shown in Fig. 5, sequences of the 5′-NCR with similar sets of mutations will be grouped together even

Table 2
Regions of the HCV Genome Used for Type and Subtype Analysis

Region	Function	Length (bases)
5′-UTR	Untranslated (non-coding)	−341
Core	Nucleocapsid	573
E1	Envelope	575
NS5B	RNA-dependent RNApolymerase	1772

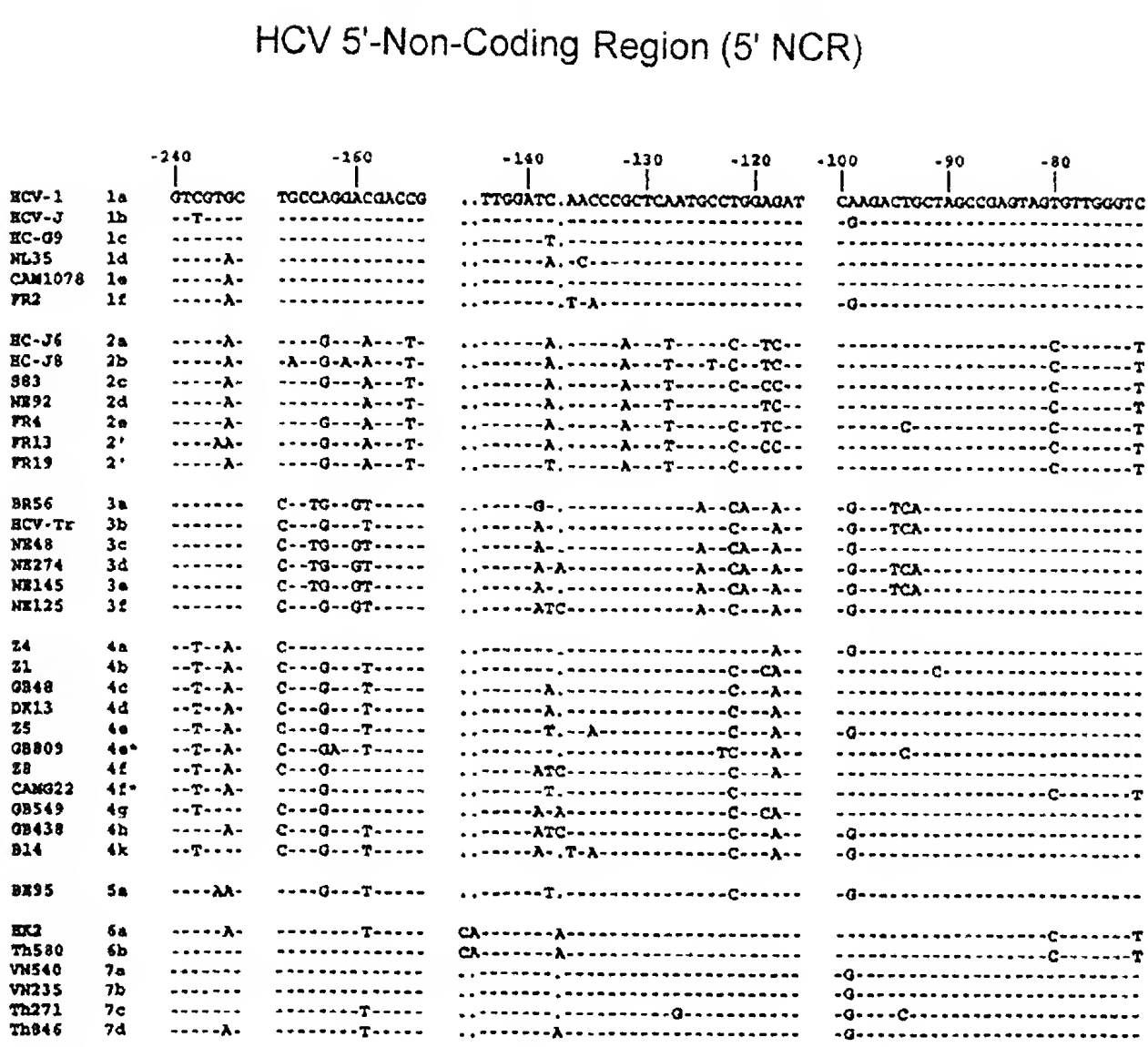

Fig. 5. The HCV 5′-Non-Coding Region (5′-NCR) of HCV. The sequences of 4 variable areas within this region are compared. Strains of the same type are grouped at the left. All sequences are compared to the type strain, HCV-1, on the top row. Dashes within a sequence indicate the same base as the type strain; dots are added to some strains to accommodate insertions in other strains. (From (Maertens and Stuyver, 1997) reprinted with permission).

though they may have some differences scattered throughout the region under consideration. Basically, the similar sets of mutations determine the 'type' and the scattered differences determine the 'subtype.'

1.6.3. Genotyping methods

The practical methodologies of viral genotyping for purposes other than drug susceptibility testing (i.e. to determine type and subtype) encompass many possibilities (Arens, 1999). Many of today's techniques are PCR-based and each has certain strengths and weaknesses. They are dependent on finding a balance between analysis of regions of the viral genome that are conserved enough to allow consistent amplification and yet sufficiently different to allow the observer to distinguish between the types and subtypes. Sequencing of various regions of the HCV genome (Table 2) is the method of choice for definitive assignment of type

and subtype but several other methods are available. Reviews of the clinical significance of HCV virus genotypes have been recently published (Mondelli and Silini, 1999). Specific methods for sequence analysis of HCV are available in primary publications (Mellor et al., 1995; Tokita et al., 1996). A convenient method, using the amplified product of a commercial qualitative PCR assay for HCV (Amplicor HCV, Roche Diagnostic Systems, Inc.) was recently published (Germer et al., 1999). With this method, the 244 bp target within the 5′-NCR was sequenced with a single anti-sense primer which yielded sequence data on a 196 bp segment. For comparison and adjudication of difficult genotypes, a 401 bp product was amplified from the NS-5 region of HCV and sequenced in both directions with sense and anti-sense primers. The authors showed that sequencing of a single region of HCV may not provide accurate genotype data. For example, 3.4% of strains classified as 1a by 5′-NCR sequence were 1b by the NS-5 sequence and 25% of those classified as 1b by 5′-NCR analysis were classified as 1a by NS-5 sequence (Germer et al., 1999). In fact, a distinction between subtypes 1a and 1b is not clinically important since all type 1's are treated similarly from a therapeutic perspective. Thus, in general, even with sequencing, which is the gold standard, it is more difficult than one might think to classify HCV strains according to subtypes.

Sequencing of HCV for the purpose of typing and subtyping is probably not the most efficient method for most clinical laboratories unless sequencing is already a daily activity in the lab. Other methods such as single-strand conformation polymorphism (SSCP) analysis (Lareu et al., 1997) and reverse hybridization (Le Pogam et al., 1998; Maertens and Stuyver, 1997) with a commercial product (LiPA; Innogenetics, Inc.) are much more convenient and user-friendly in the lab.

1.7. CMV

1.7.1. Antiviral drugs

CMV infections are a major cause of morbidity and mortality among patients who are immunocompromised due to organ transplant or HIV infection. There are three drugs currently approved for treatment of CMV infections, gangiclovir (GCV), foscarnet and cidofovir (Fig. 6).

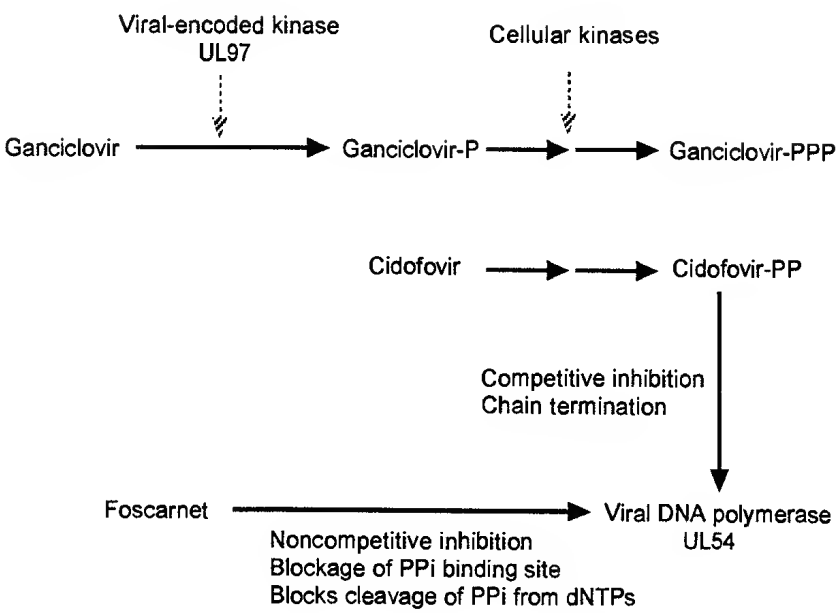

Fig. 6. Mechanism of action of antiviral agents against CMV. The three anti-CMV drugs ganciclovir, cidofovir and foscarnet are processed in different ways and all exert their effect on the viral DNA polymerase, the product of the viral UL54 gene. (Adapted from (Erice, 1999a) reprinted with permission).

GCV requires phosphorylation to the active form by the viral-coded phosphotransferase (UL97) which, in the presence of certain mutations, does not perform this activity efficiently. Foscarnet requires no activation and cidofovir is activated by cellular enzymes. All three drugs inhibit the viral DNA polymerase (UL54) through various mechanisms. Thus, these two viral genes may mutate at specific positions to confer resistance to any or all of these drugs and detection of resistance is important in deciphering the lack of a therapeutic response. Genotyping of UL97 by sequencing is a reasonable undertaking since resistance mutations are within a region of about 150 codons or 450 base pairs. Sequencing of the UL54 gene is much more cumbersome since resistance-conferring mutations occur over a region of about 600 codons or 1800 base pairs. Mutation detection in the UL97 gene detects the vast majority of GCV resistant viruses (Erice, 1999b).

There are volumes and years of clinical experience with GCV and it remains the front line drug for treatment. There is much less clinical experience with foscarnet and cidofovir but both are receiving attention as useful drugs for GCV-resistant strains of CMV. GCV is activated to the monophosphate in infected cells by the viral product of the UL97 gene (phosphotransferase) and then further activated to the triphosphate by cellular enzymes. Fully activated GCV is a competitive inhibitor of the DNA polymerase (UL54 gene) and also causes a slowing and then cessation of chain elongation. Foscarnet requires no activation and interacts directly with the viral DNA polymerase. Cidofovir is already in monophosphate form and is activated by cellular enzymes to the diphosphate form which is a competitive inhibitor and, when incorporated by DNA polymerase, causes slow chain growth and termination of synthesis. Resistance may be conferred to GCV by mutations in the UL97 gene and to all three drugs by mutations in the UL54 gene. In one study (Smith et al., 1998), resistance-associated UL97 mutations were detected in 89% of the GCV-resistant isolates. UL97 mutations were associated with both low- and high-level GCV resistance but UL97 mutations alone do not appear to modulate the level of this resistance. Thus, the presence of UL97 mutations clearly identifies a clinically resistant isolate but the level of resistance can only be discerned with a UL54 genotypic analysis or a phenotype assay. Length of treatment is critical for this conversion to high-level resistance. During the first 9 months of treatment, about 20% of GCV-resistant isolates displayed high-level resistance whereas after >9 months of treatment 64% of isolates were high-level resistant (Smith et al., 1998). In this same study, one of 28 isolates (4%) displayed phenotypic low level GCV resistance but had no detectable mutations in UL97. We have also seen this phenomenon in some isolates from our lab in which low level resistance by plaque reduction assay (PRA) is not reflected in the genotype.

1.7.2. Sequencing methods

A method for PCR amplification and sequencing of a portion of the CMV UL97 gene is shown in Fig. 7. The primers were adapted from Wolf et al. (1995), such that the PCR product shown here encompasses the region containing all known GCV mutations (codons 400 to ~ 620). Sequencing primers were chosen so as to obtain a sequence in both directions over the entire PCR product. Total DNA purified from a 1–2+ positive tube culture (i.e. 25–50% of the cells are showing viral cytopathic effect, CPE) of CMV is the PCR target of choice for this procedure. However, since CMV grows very slowly it may take a few weeks to get to this stage. We have employed a nested PCR reaction using the above CMV1F and CMV6R primers as the outside pair and the primers CMV3F and CMV7FR as the inside pair to amplify a product directly from DNA extracted from whole blood. This technique works if the CMV viral load in the patient blood is high. With this nested PCR method, one can bypass the culture altogether and obtain a product for sequencing within a few days. Others (Liu et al., 1998) have used nested PCR to amplify the UL97 gene from vitreous fluid of patients undergoing antiviral therapy for CMV retinitis.

1.7.3. Advantages

One major advantage of genotyping the UL97 gene for detection of antiviral resistance in CMV

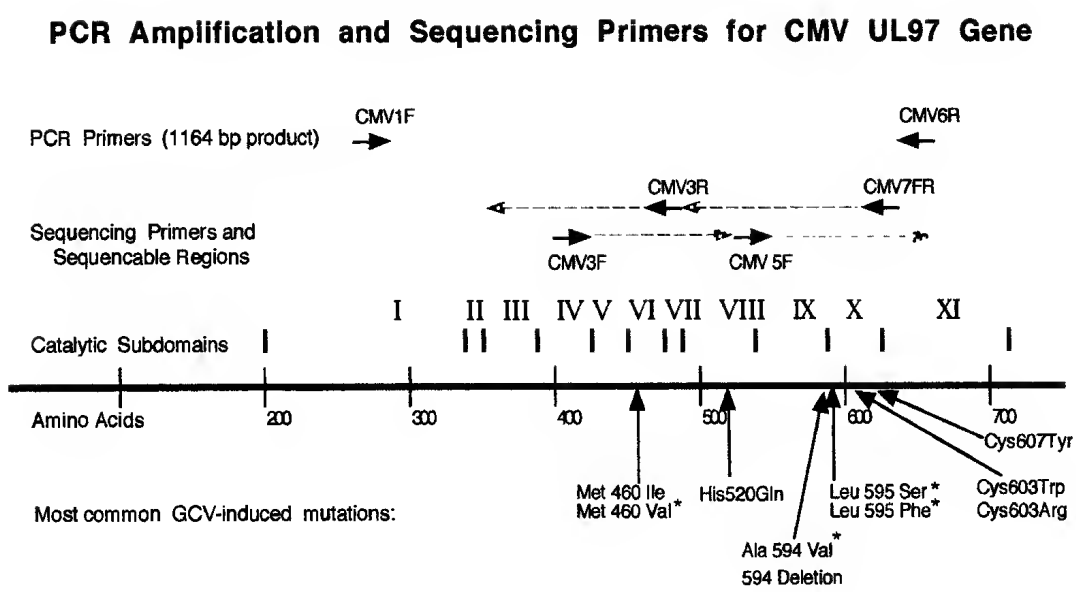

Fig. 7. PCR amplification and sequencing primers for CMV UL97 gene. The catalytic subdomains of the enzyme are shown in Roman numerals. Primers used for PCR amplification are CMV1F and CMV6R and primers used for sequencing are CMV3R, CMV7FR, CMV3F and CMV5F. All primers are from Wolf et al. (1995). The most common ganciclovir-induced mutations and their approximate locations are shown. Mutations with asterisks are those detected by the PCR/REN assay described by Chou et al. (1995).

is that it can usually be performed more quickly than a PRA. CMV may require 1–4 weeks to reach 1–2+ CPE in culture. Then extraction of DNA, amplification and sequencing reactions can be performed in 2 days, the sequencing gel can be run on a third day and results analyzed on day 4. This scenario is impractical for small numbers of specimens but becomes efficient if 5–10 specimens can be run at once. In contrast to this, a plaque reduction assay with CMV may require 6–8 weeks, but may be more efficient with small numbers of samples. Additionally, sequencing has the advantage of providing information for the entire gene so as to detect all mutations whereas other methods may only detect mutations at specific codons (Chou et al., 1995; Hanson et al., 1995).

1.8. HIV

1.8.1. Mutations

The development of antiretroviral drug resistance is currently a significant cause of treatment failures in HIV-infected patients (Hirsch et al., 1998, 2000) and genotyping for detection of mutations that confer resistance has become the standard in the treatment of viral breakthrough. Since there are only three classes of antiretroviral drugs, cross-resistance within a class may limit the options for alternative regimens. Because of the lack of proofreading activity in the RT enzyme that copies the genome of HIV, the virus exists in infected individuals as a population of variants or quasispecies (Delwart et al., 1993, 1997). This results in the random appearance and subsequent selection of resistant mutants in the presence of a selection pressure such as an inhibitory drug. Initially, a single mutation may occur, confer a low level of resistance and soon predominate in the population. With ongoing replication even at a fairly low level, additional mutations that confer high level resistance soon appear and the population as a whole becomes highly resistant (Martinez-Picado et al., 1999). For some drugs (e.g. 3TC and nevirapine) a single mutation confers high level resistance. Mutations that directly affect the binding of drug to the target enzyme are called primary mutations. Secondary mutations are those that accumulate to confer higher level resistance or to enhance viral fitness in the presence of the drug. Secondary mutations do not

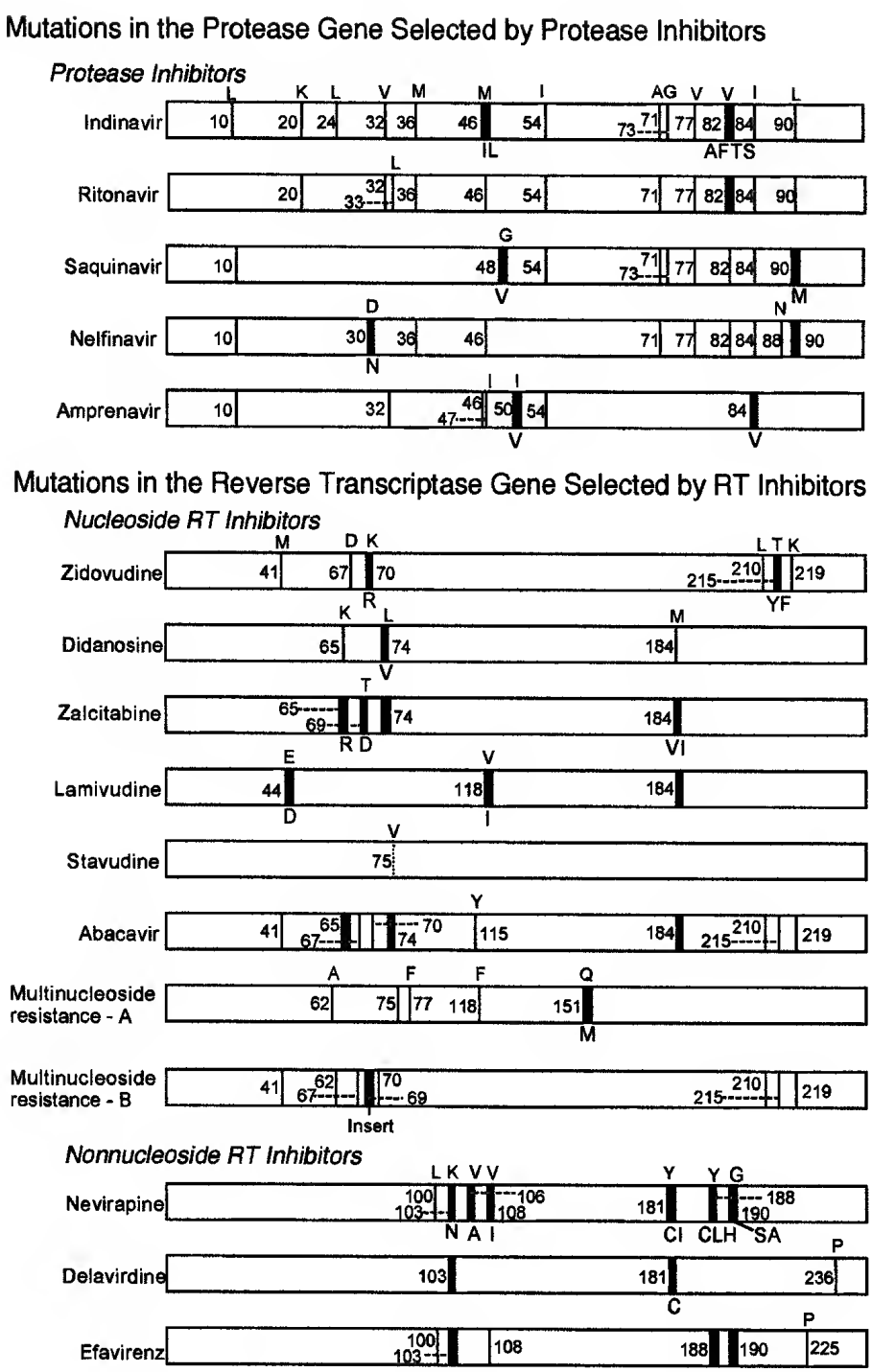

Fig. 8. Primary and secondary mutations in protease and reverse transcriptase (RT) of HIV that affect resistance to antiretroviral drugs. Dark bars represent primary mutations, light bars represent secondary mutations and the dotted bar represents a mutation selected in vitro but is rarely seen in clinical isolates. Primary mutations are the first to appear and usually result in decreased substrate binding. Secondary mutations generally do not confer resistance in the absence of primary mutations for the same drug. Numbers within the bars refer to the codon positions in the protease or RT gene. The letters above the bars indicate the amino acid (one-letter code) at that position in wild-type virus and the letters below the bars indicate the common amino acid substitutions associated with the primary mutation at that site. Primary mutations in RT for multinucleoside resistance include Q151M and a 2 amino acid insert following codon 69. From Hirsch et al ((Hirsch et al., 2000) reprinted with permission).

usually confer resistance in the absence of primary mutations for the same drug (Hirsch et al., 2000). Fig. 8 lists the common primary and secondary mutations that affect resistance to antiretroviral drugs directed against the protease gene or the RT gene.

1.8.2. Sequencing methods

Because of the intense interest in monitoring the development of resistance during the course of therapy for HIV disease (Weinstock et al., 2000; Shafer et al., 1998; Yahi et al., 1999; Hirsch et al., 2000; Rodes et al., 2000), methods have evolved quickly for detection of mutations. As with some of the other viruses discussed above, both genotypic and phenotypic methods are currently available. Since there are numerous mutations that can confer resistance to the currently 15 approved drugs, sequencing is the most efficient means to detect all of them. Genotypic assays have been developed by several non-commercial labs ('home-brew' assays) and by two commercial companies, Perkin Elmer/Applied Biosystems, Inc (PE/ABI) and Visible Genetics, Inc (VisGen). All of these systems rely on initial extraction of viral RNA from patient plasma, RT-PCR of the extracted RNA to amplify about 1500 bp of protease and RT and then a sequencing reaction with dye-terminators (PE/ABI) or dye-labelled primers (VisGen). Both companies make sequencing instruments for electrophoresis of the samples and both provide software to assemble the segments and align the assembled patient sequence with a reference sequence to aid in the identification of mutant codons. The PE/ABI primers for PCR and sequencing are shown in Fig. 9.

In the PE/ABI system, Amp1 and Amp2 are the primers for PCR and the sequencing primers are A–D and F–H. Theoretically, this system provides double coverage at a minimum for the entire 1.5 Kbp of interest and quadruple coverage may be obtained for certain regions. The PE/ABI software combines the functions of several previously-available software packages to trim the sequence segments, generate a 'contig' (the contiguous consensus sequence that is generated by assemblage of the overlapping individual segments) and align the contig with a reference sequence (pNL4-3). Then the manual part of the procedure begins, in which a real person toggles through the entire sequence and edits (confirm or override) the computer base calls and also makes the final decision about polymorphic sites where a mutant base might constitute a minor proportion in the background of wild-type base. The ABI software prints a complete mutation report which identifies 'reported' and 'novel' mutations based on the Los Alamos HIV database.

The VisGen genotyping kit may be used in conjunction with a number of extraction procedures. The purified viral RNA is amplified in a single-tube RT-PCR step, the product of which need not be purified but can be used directly in the subsequent sequencing reactions. The CLIP™ methodology is a proprietary chemistry employing four pairs of sequencing primers, the upstream primer of each pair is labelled with one dye and the downstream primer of each pair is labelled

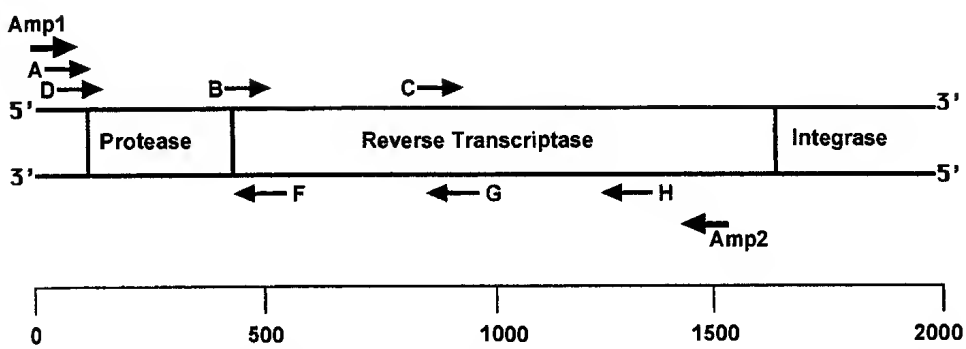

Fig. 9. ABI PCR and sequencing primers for HIV genotyping. PCR primers are Amp1 and Amp2 which generate a product of about 1500 bp. Sequencing primers A–D, and F–H are used to sequence in both directions across the entire PCR product.

with another dye. Each primer pair is present in each of 4 dideoxy-terminator reactions, one reaction for each of the four dideoxy bases. Thus, this arrangement requires 16 different reactions and 16 lanes on the sequencing gel in order to cover the HIV protease and RT genes in both directions. The data is analyzed with the VisGen software and is interpreted to provide a resistance report.

According to some recent studies done to compare results from different labs, the manual editing is the weak link in the procedure and the step at which numerous hours of experience in reading sequences pays off with regards to overall accuracy. Thus, there is some lab-to-lab variability in the accuracy of HIV genotype results. In a recent worldwide survey (Schuurman et al., 1999), 90% of the 23 participating labs correctly identified mutations at codons containing only mutant virus or only wild type virus, but only about 50% of the labs were able to correctly identify mutations where the mutant represented only 25% of the DNA population at that codon.

1.8.3. Genotype and interpretation

The final analysis of all relevant codons (see Fig. 8) should be recorded as edited within the software and on a separate sheet for transmission to physicians (example in Fig. 10). In both commercial packages the software will generate a written report. Whether the lab should generate a report listing mutations and also providing interpretation with regards to possible drug resistance is open to discussion. The HIV genotype is most useful for determining which drugs will not be effective in ongoing treatment. The decision as to which drugs may be useful for future treatment is dependent on the patient treatment history (including viral load and CD4 counts), the order in which drugs have previously been used for treatment and the extent of treatment failure (breakthrough viral load). Interpretation of drug resistance in the context of many complex variables is difficult (Hirsch et al., 1998, 2000).

1.8.4. Cost analysis

Commercial products for genotyping of HBV and CMV are unavailable. Home-brew PCR amplification and cycle sequencing using 4 primers (2 forward and 2 reverse) as shown in Fig. 7 for the UL97 gene of CMV, costs only a few dollars for PCR and about $6–8 per primer for the sequencing reagents (i.e. primer plus enzyme plus dye-terminators). Sequencing core labs charge $8–12 per lane for the gel run. Thus, in this example, a good sequence (i.e. both directions) of 600–700 bases using ABI dye-terminator reagents run in a core lab on ABI instruments would cost about $60–85 not including any instrument purchases, maintenance or amortized costs nor any technician labor costs. Sequencing a shorter region (e.g. 440 bases in domains B and C of HBV polymerase or 244 bases of the 5′-NCR of HCV) would cost proportionately less.

Commercial kits for viral genotyping by sequencing are available only for HIV. Several home-brew methods have been published (Condra et al., 1995; Larder et al., 1995; Nijhuis et al., 1997) (Condra et al., Nature 1995; Nijhuis et al., J Infect Dis 1997; Larder et al., Science 1995), but these are in no way 'standardized' and no commercial computer software exists to generate the contig, align to a reference sequence and identify codons associated with drug resistance all in one continuous process. The cost of an HIV genotype analysis using an ABI commercial kit is about $170 per patient specimen for kit reagents (convenience is not cheap and price schedules may vary depending on the number of tests the lab runs) and another $60–70 for the gel lanes if 6–7 sequencing primers are used. For VisGen reagents the cost is $220 per patient specimen and this includes the sequencing instrument, dedicated computer and software through a reagent rental agreement. Additional costs include extraction reagents for the VisGen procedure (which are not included in the kit) and the labor costs associated with setting up and running the gels locally or, for the ABI procedure, the cost of the HIV Genotype software and possibly a computer on which to run it. Thus, the total cost of an HIV genotype with either kit will be in the range of $310–380 depending on labor costs, lab specimen volume and general efficiency of performing very complex, multi-step procedures and analyses. Typical charges to the patient for this test are in the range of $400–500.

RA032300 **HIV-1 Protease/Reverse Transcriptase Genotype**

Patient Name: Specimen Date: 3/23/00 Physician:

Protease

Codon	Wild Type Codon	Mutant Codon	Primary Mutations	Secondary Mutations	% Mutant
10	CTC	ATC ✓ CGC GTC		L10I ✓ L10R L10V	100
20	AAG	ATG AGG		K20M K20R	0
24	TTA	ATA		L24I	0
30	GAT	AAT	**D30N**		0
32	GTA	ATA GTG		V32I	0
33	TTA	TTC		L33F	0
36	ATG	ATA ✓		M36I ✓	100
46	ATG	ATA TTG	**M46I** **M46L**		0
47	ATA	CTA		I47V	0
48	GGG	GTG	**G48V**		0
50	ATT	GTT	**I50V**		0
54	ATC	GTC ✓ ACC CTC		I54V ✓ I54T I54L	100
63	CTC	CCC ✓ CAC CAA		L63P ✓ L63H L63Q	100
71	GCT	GTT ACT		A71V A71T	0
73	GGT	AGT		G73S	0
77	GTA	ATA		V77I	0
82	GTC	GCC ✓ TTC ACC	V82A ✓ **V82F** **V82T**		100
84	ATA	GTA	**I84V**		0
88	AAT	GAT ATG		N88D N88M	0
90	TTG	ATG	**L90M**		0

RT

Codon	Wild Type Codon	Mutant Codon	Primary Mutations	Secondary Mutations	% Mutant
41	ATG	TTG ✓ CTG		M41L ✓	100
62	GCC	GTC		A62V	0
65	AAA	AGA	**K65R**		0
67	GAC	AAC ✓		D67N ✓	100
69	ACT	GAT ✓ AAT		T69D ✓ T69N	100
70	AAA	AGA	**K70R**		0
74	TTA	GTA ATA	**L74V** **L74I**		0
75	GTA	ACA GCA ATG		V75T V75A V75M	0
77	TTC	TTA TTG		F77L	0
100	TTA	ATA		L100I	0
103	AAA	AAC AAT	**K103N**		0
106	GTA	GCA	**V106A**		0
108	GTA	ATA	**V108I**		0
115	TAT	TTT		Y115F	0
116	TTT	TAT		F116Y	0
151	CAG	ATG	**Q151M**		0
181	TAT	TGT ATT	**Y181C** **Y181I**		0
184	ATG	ATA ACG ✓ GTG or GTA	**M184I** **M184T** **M184V** ✓		100
188	TAT	TGT TTA CTT	**Y188C** **Y188L** ✓		100
190	GGA	GCA AGT	**G190A** **G190S**		0
210	TTG	TGG ✓		L210W ✓	100
215	ACC	TAC ✓ TTC	**T215Y** ✓ **T215F**		100
219	AAA	CAA AAG ✓ AAC		K219Q K219N	0

Bregt

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mA 4/4/00

Secondary mutations generally do not confer resistance in the absence of primary mutations for the same drug. The reagents used for this HIV-1 genotype determination are not approved by the FDA and thus for Research Use Only.

Fig. 10. Example of an HIV genotype report. This report provides genotype data on 20 protease codons and 23 reverse transcriptase (RT) codons. Primary and secondary mutations are denoted in separate columns on the report. If a mutant population at a particular codon is less than 100%, it is denoted as such in the column headed '% Mutant' (for example the mutant might comprise only 50% of the population at a particular codon).

1.8.5. Can clinical virology labs perform sequence-based genotyping?

Any clinical virology lab that has a good foundation in molecular techniques such as nucleic acid purification, PCR primer design, optimization of PCR reactions and gel analysis of PCR products has the basis from which to launch a viral genotyping program. The next step, and it's a pretty big step, is the actual sequencing itself. It is helpful in the long run if somebody in the lab becomes familiar with the entire process of sequencing, purification of the sequencing products, preparation of the gels, setting up the instrument, loading the samples. However, this is not necessary as many universities and research institutes have core sequencing labs that perform much of this 'routine' work for a price. Once the sequencing products are made, they can be sent to the core lab and 2 days later the sequence electropherograms may be downloaded over a computer network and analyzed on a Virology Lab computer. The learning curve for the analysis step is long and steep but is certainly within the grasp of a good technologist with the interest and aptitude to develop the skill necessary to peruse the profiles and carefully and patiently edit the electropherograms.

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References

Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 1998;27:1670–7.

Arens M. Methods for subtyping and molecular comparison of human viral genomes. Clin Microbiol Rev 1999;12:612–26.

Aye TT, Bartholomeusz A, Shaw T, Bowden S, Breschkin A, McMillan J, Angus P, Locarnini S. Hepatitis B virus polymerase mutations during antiviral therapy in a patient following liver transplantation. J Hepatol 1997;26:1148–53.

Benhamou Y, Bochet M, Thibault V, Di Martino V, Caumes E, Bricaire F, Opolon P, Katlama C, Poynard T. Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. Hepatology 1999;30:1302–6.

Boyer N, Marcellin P. Pathogenesis, diagnosis and management of hepatitis C. J Hepatol 2000;32:98–112.

Castro FJ, Esteban JI, Sauleda S, Viladomiu L, Dragon EA, Esteban R, Guardia J. Utility of early testing for HCV viremia as predictive factor of sustained response during interferon or interferon plus ribavirin treatment. J Hepatol 2000;32:843–9.

Chambers J, Angulo A, Amaratunga D, Guo H, Jiang Y, Wan JS, Bittner A, Frueh K, Jackson MR, Peterson PA, Erlander MG, Ghazal P. DNA microarrays of the complex human cytomegalovirus genome: profiling kinetic class with drug sensitivity of viral gene expression. J Virol 1999;73:5757–66.

Chou S, Erice A, Jordan MC, Vercellotti GM, Michels KR, Talarico CL, Stanat SC, Biron KK. Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J Infect Dis 1995;171:576–83.

Condra JH, Schleif WA, Blahy OM, Gabryelski LJ, Graham DJ, Quintero JC, Rhodes A, Robbins HL, Roth E, Shivaprakash M, et al. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature 1995;374:569–71.

Delwart EL, Pan H, Sheppard HW, Wolpert D, Neumann AU, Korber B, Mullins JI. Slower evolution of human immunodeficiency virus type 1 quasispecies during progression to AIDS. J Virol 1997;71:7498–508.

Delwart EL, Shpaer EG, Louwagie J, McCutchan FE, Grez M, Rubsamen-Waigmann H, Mullins JI. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science 1993;262:1257–61.

Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256–63.

Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′-thiacytidine and related analogues. Proc Natl Acad Sci USA 1991;88:8495–9.

Erice A. Antiviral susceptibility testing. In: Storch G, editor. Essentials of Diagnostic Virology. New York, NY: Churchill-Livingstone, 1999a:272–89.

Erice A. Resistance of human cytomegalovirus to antiviral drugs. Clin Microbiol Rev 1999b;12:286–97.

Fontaine H, Thiers V, Pol S. Hepatitis B virus genotypic resistance to lamivudine letter. Ann Intern Med 1999;131:716–7.

Ganem D. *Hepadnaviridae* and their replication. In: Fields BN, Knipe DM, Howley PM, editors. Fields Virology, vol. 2. Philadelphia: Lippincott-Raven, 1996:2703–37.

Germer JJ, Rys PN, Thorvilson JN, Persing DH. Determination of hepatitis C virus genotype by direct sequence analysis of products generated with the Amplicor HCV test. J Clin Microbiol 1999;37:2625–30.

Gunther S, von Breunig F, Santantonio T, Jung MC, Gaeta GB, Fischer L, Sterneck M, Will H. Absence of mutations in the YMDD motif/B region of the hepatitis B virus polymerase in famciclovir therapy failure. J Hepatol 1999;30:749–54.

Hanson MN, Preheim LC, Chou S, Talarico CL, Biron KK, Erice A. Novel mutation in the UL97 gene of a clinical cytomegalovirus strain conferring resistance to ganciclovir. Antimicrob Agents Chemother 1995;39:1204–5.

Hirsch MS, Brun-Vezinet F, D'Aquila RT, Hammer SM, Johnson VA, Kuritzkes DR, Loveday C, Mellors JW, Clotet B, Conway B, Demeter LM, Vella S, Jacobsen DM, Richman DD. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. J Am Med Assoc 2000;283:2417–26.

Hirsch MS, Conway B, D'Aquila RT, Johnson VA, Brun-Vezinet F, Clotet B, Demeter LM, Hammer SM, Jacobsen DM, Kuritzkes DR, Loveday C, Mellors JW, Vella S, Richman DD. Antiretroviral drug resistance testing in adults with HIV infection: implications for clinical management. International AIDS Society — USA Panel. J Am Med Assoc 1998;279:1984–91.

Honkoop P, Niesters HG, de Man RA, Osterhaus AD, Schalm SW. Lamivudine resistance in immunocompetent chronic hepatitis B. Incidence and patterns. J Hepatol 1997;26:1393–5.

Korba BE, Boyd MR. Penciclovir is a selective inhibitor of hepatitis B virus replication in cultured human hepatoblastoma cells. Antimicrob Agents Chemother 1996;40:1282–4.

Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61–8.

Larder BA, Kemp SD, Harrigan PR. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science 1995;269:696–9.

Lareu RR, Swanson NR, Fox SA. Rapid and sensitive genotyping of hepatitis C virus by single-strand conformation polymorphism. J Virol Methods 1997;64:11–8.

Le Pogam S, Dubois F, Christen R, Raby C, Cavicchini A, Goudeau A. Comparison of DNA enzyme immunoassay and line probe assays (Inno-LiPA HCV I and II) for hepatitis C virus genotyping. J Clin Microbiol 1998;36:1461–3.

Lee NY, Tang Y, Espy MJ, Kolbert CP, Rys PN, Mitchell PS, Day SP, Henry SL, Persing DH, Smith TF. Role of genotypic analysis of the thymidine kinase gene of herpes simplex virus for determination of neurovirulence and resistance to acyclovir [published erratum appears in J. Clin. Microbiol. 2000 Mar;38:1311. J. Clin. Microbiol. 1999;37, 3171–3174.

Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733–45.

Liu W, Kuppermann BD, Martin DF, Wolitz RA, Margolis TP. Mutations in the cytomegalovirus UL97 gene associated with ganciclovir-resistant retinitis. J Infect Dis 1998;177:1176–81.

Maertens G, Stuyver L. Genotypes and genetic variation of hepatitis C virus. In: Harrison TJ, Zuckerman AJ, editors. The Molecular Medicine of Viral Hepatitis. Sommerset, NJ: John Wiley & Sons, Ltd, 1997:183–233.

Main J, Brown JL, Howells C, Galassini R, Crossey M, Karayiannis P, Georgiou P, Atkinson G, Thomas HC. A double blind, placebo-controlled study to assess the effect of famciclovir on virus replication in patients with chronic hepatitis B virus infection. J Viral Hepat 1996;3:211–5.

Martinez-Picado J, Savara AV, Sutton L, D'Aquila RT. Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. J Virol 1999;73:3744–52.

McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 1998;339:1485–92.

Mellor J, Holmes EC, Jarvis LM, Yap PL, Simmonds P. Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. The International HCV Collaborative Study Group. J Gen Virol 1995;76:2493–507.

Mondelli MU, Silini E. Clinical significance of hepatitis C virus genotypes. J Hepatol 1999;31:65–70.

Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science 1998;282:103–7.

Nijhuis M, Schuurman R, de Jong D, van Leeuwen R, Lange J, Danner S, Keulen W, de Groot T, Boucher CA. Lamivudine-resistant human immunodeficiency virus type 1 variants 184V require multiple amino acid changes to become co-resistant to zidovudine in vivo. J Infect Dis 1997;176:398–405.

Oldenburg MC, Siebert M. New cleavase fragment length polymorphism method improves the mutation detection assay. Biotechniques 2000;28:351–7.

Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection

with hepatitis C virus. International Hepatitis Interventional Therapy Group IHIT. Lancet 1998;352:1426–32.

Poynard T, McHutchison J, Goodman Z, Ling MH, Albrecht J. Is an a la carte combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Project Group. Hepatology 2000;31:211–8.

Roche B, Samuel D, Gigou M, Feray C, Virot V, Majno P, Serraf L, David MF, Dusseaix E, Reynes M, Bismuth H. Long-term ganciclovir therapy for hepatitis B virus infection after liver transplantation. J Hepatol 1999;31:584–92.

Rodes B, Holguin A, Soriano V, Dourana M, Mansinho K, Antunes F, Gonzalez-Lahoz J. Emergence of drug resistance mutations in human immunodeficiency virus type 2-infected subjects undergoing antiretroviral therapy. J Clin Microbiol 2000;38:1370–4.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463–7.

Schlesinger Y, Tebas P, Gaudreault-Keener M, Buller RS, Storch GA. Herpes simplex virus type 2 meningitis in the absence of genital lesions: improved recognition with use of the polymerase chain reaction. Clin Infect Dis 1995;20:842–8.

Schuurman R, Demeter L, Reichelderfer P, Tijnagel J, de Groot T, Boucher C. Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase. J Clin Microbiol 1999;37:2291–6.

Shafer RW, Winters MA, Palmer S, Merigan TC. Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients. Ann Intern Med 1998;128:906–11.

Shaw T, Mok SS, Locarnini SA. Inhibition of hepatitis B virus DNA polymerase by enantiomers of penciclovir triphosphate and metabolic basis for selective inhibition of HBV replication by penciclovir. Hepatology 1996;24:996–1002.

Smith IL, Cherrington JM, Jiles RE, Fuller MD, Freeman WR, Spector SA. High-level resistance of cytomegalovirus to ganciclovir is associated with alterations in both the UL97 and DNA polymerase genes [published erratum appears in J. Infect. Dis. 1998 Apr;177(4):1140–1141. J. Infect. Dis. 1997;176, 69–77.

Takayama M, Takayama N, Inoue N, Kameoka Y. Application of long PCR method of identification of variations in nucleotide sequences among varicella-zoster virus isolates. J Clin Microbiol 1996;34:2869–74.

Thibault V, Benhamou Y, Seguret C, Bochet M, Katlama C, Bricaire F, Opolon P, Poynard T, Agut H. Hepatitis B virus HBV mutations associated with resistance to lamivudine in patients coinfected with HBV and human immunodeficiency virus. J Clin Microbiol 1999;37:3013–6.

Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 1996;24:714–7.

Tokita H, Okamoto H, Iizuka H, Kishimoto J, Tsuda F, Lesmana LA, Miyakawa Y, Mayumi M. Hepatitis C virus variants from Jakarta, Indonesia classifiable into novel genotypes in the second (2e and 2f), tenth (10a) and eleventh 11a genetic groups. J Gen Virol 1996;77:293–301.

Trepo C, Jezek P, Atkinson G, Boon R, Young C. Famciclovir in chronic hepatitis B: results of a dose-finding study. J Hepatol 2000;32:1011–8.

Weinstock H, Respess R, Heneine W, Petropoulos CJ, Hellmann NS, Luo CC, Pau CP, Woods T, Gwinn M, Kaplan J. Prevalence of Mutations Associated with Reduced Antiretroviral Drug Susceptibility among Human Immunodeficiency Virus Type 1 Seroconverters in the United States, 1993–1998. J Infect Dis 2000;182:330–3.

Wolf DG, Smith IL, Lee DJ, Freeman WR, Flores-Aguilar M, Spector SA. Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J Clin Invest 1995;95:257–63.

Wolters LM, Honkoop P, Niesters HG, de Man RA. Efficacy of famciclovir treatment in chronic hepatitis B patients with different mutations at position 552 of the DNA polymerase gene [letter]. J Hepatol 1998;28:909–10.

Xiong X, Yang H, Westland CE, Zou R, Gibbs CS. In vitro evaluation of hepatitis B virus polymerase mutations associated with famciclovir resistance. Hepatology 2000;31:219–24.

Yahi N, Tamalet C, Tourres C, Tivoli N, Ariasi F, Volot F, Gastaut JA, Gallais H, Moreau J, Fantini J. Mutation patterns of the reverse transcriptase and protease genes in human immunodeficiency virus type 1-infected patients undergoing combination therapy: survey of 787 sequences. J Clin Microbiol 1999;37:4099–106.

Zou S. A practical approach to genetic screening for influenza virus variants. J Clin Microbiol 1997;35:2623–7.

Zoulim F, Trepo C. Drug therapy for chronic hepatitis B: antiviral efficacy and influence of hepatitis B virus polymerase mutations on the outcome of therapy. J Hepatol 1998;29:151–68.